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Introduction

The goal of the research is to develop a magnetic nanoparticle MR contrast targeted to the gastrin releasing peptide receptor (GRP receptor) that will be used to image the intra-prostatic distribution of this key molecular marker. Key accomplishments during year 1 were:

1. Synthesis of a variety peptides interaction with the GRP receptor.
2. Demonstration the GRP receptor prefers highly soluble positively charged BN-like peptides.
3. Preliminary preparation of peptide-nanoparticle conjugates.
4. Development of a FITC based assay.

Research conducted to date

I. Synthesis and chemical characterization of bombesin-like peptides

Our goal is to synthesize a highly soluble peptide with high binding affinity for the GRP receptor. High solubility is needed because at numbers of the peptide per nanoparticle, the insolubility of the GRP binding amino acids causes the nanoparticle to precipitate. Peptides synthesized have shorter 8 amino acid or longer 14 sequences of amino acids binding the GRP receptor. It's well known that the C terminus of the peptide is essential to binding to the GRP receptor, but the number of residues required for high affinity binding is unclear. Therefore, two series of peptides were synthesized, one containing the 8 C terminal amino acids of bombesin, the other containing the first 14.

Peptides have different solubilizing sequences of d-amino acids which serve as linkers between the C-terminal sequences that interact with the GRP receptor and N-terminal cysteine that is attached to CLIO. It was also desired to have peptides more hydrophilic than bombesin for solubility reasons. Conjugation a hydrophobic peptide to nanoparticles at high numbers of peptide per nanoparticle has proven difficult due to the precipitation of the conjugate in biological media.

All peptides were synthesized using standard FMOC chemistry using Rink amide resin, and cleaved with reagent R. They were then purified by HPLC, quantified by HPLC using tryptophan and histidine as internal standards, and characterized by mass spectroscopy. All peptides were characterized by mass spec and were within 1 dalton of theoretical weights.

Table 1: Sequence of bombesin-like peptides synthesized Peptides are identified by designation in parentheses.	
Short Peptides	Long Peptides
(S-G) CGQWAVGHLM-NH ₂	(L-G) CGQRLGNQWAVGLHLM-NH ₂
(S-ah) CεQWAVGHLM-NH ₂	(L-ah) CεQRLGNQWAVGLHLM-NH ₂
(S-s) CsssQWAVGHLM-NH ₂	(L-s) CsssGQRLGNQWAVGLHLM-NH ₂
(S-r) CrrrQWAVGHLM-NH ₂	(L-r) CrrrGQRLGNQWAVGLHLM-NH ₂
(S-k) CkkkQWAVGHLM-NH ₂	(L-k) CkkkGQRLGNQWAVGLHLM-NH ₂
(S-k) CeeeQWAVGHLM-NH ₂	(L-e) CeeeGQRLGNQWAVGLHLM-NH ₂
(S-r-FITC-1) CK(FITC)rrrQWAVGHLM-NH ₂	
(S-r-FITC-2) K(FITC)rrrQWAVGHLM-NH ₂	

ε=aminohexanoic acid

lower case letters are the d-isomer of the amino acid

II. Pharmacological characterization of bombesin like peptides.

We have evaluated peptides for interaction with the GRP receptor using a commercially available radioactive bombesin and GRP receptor bearing cells.

EC50s for the various bombesin derivatives vs. [¹²⁵I-Tyr²] bombesin:

Table 2: Pharmacological Activity of peptides			
Peptide Sequ		Spacer characteristics	EC50 (nM)
Short peptides			
S-g	Glycine	Hydrophobic	21
S-ah	Aminohexanoic acid	Hydrophobic	7.5
S-s	Serine	Hydrophilic, noncharged	11
S-r	Arginine	Hydrophilic, cationic	3.8
S-k	Lysine	Hydrophilic, cationic	8.5
G-e	Glutamic acid	Hydrophilic, anionic	180
S-r-FITC-1	Arginine	Hydrophilic, cationic	3.9
Long peptides			
L-g	Glycine	Hydrophobic	9.6
L-ah	Aminohexanoic acid	Hydrophobic	11
L-s	Serine	Hydrophilic, noncharged	8.0
L-r	Arginine	Hydrophilic, cationic	3.4
L-k	Lysine	Hydrophilic, cationic	3.0
L-e	Glutamic acid	Hydrophilic, anionic	52
Control			
Bombesin	No spacer		4.4

The general trend is that the longer bombesin peptides have better affinity for the receptor than the peptides with less of the bombesin sequence. The effect of the spacer is that negatively charges are strongly detrimental to the binding, while hydrophobic spacers are mildly detrimental. Positively charged spacers have the strongest binding. This suggests that the binding pocket for the gastrin releasing protein receptor is located in an area of negative charge.

III. Synthesis of peptide-nanoparticle conjugates

We have synthesized peptide-nanoparticle conjugates with different numbers of peptides per particle (Table 3). Amino CLIO (a crosslinked dextran coated iron oxide nanoparticle with attached amino groups) was reacted with excess SPDP (succinimidyl 3-(2pyridyldithio) propionate) to form a nanoparticle reactive to cysteine. This was reacted with sufficient peptide to add the desired number of peptides per nanoparticle, linked via a disulfide bond. A total of nine peptide-CLIO conjugates were synthesized. They have not been evaluated for pharmacological activity.

Table 3: Bombesin-like peptide-CLIO conjugates synthesized:	
Peptide Sequence And Designation	Nominal loading (peptides/nanoparticle)
CGQRLGNQWAVGLHLM-NH ₂ (L-g)	1, 3, 10
CrrrGQRLGNQWAVGLHLM-NH ₂ (L-r)	1, 3, 10
CeeeGQRLGNQWAVGLHLM-NH ₂ (L-e)	1, 3, 10

IV. Development of FITC based assay

For reasons of convenience, we found it desirable to develop an assay that avoided the use of a radiolabeled tracer. This is a competition assay, where the sample is mixed with an HRP labeled antibody for FITC on a plate labeled with a known amount of FITC. We've shown that the antibody does not bind to other dyes or components in growth media, and is specific for either FITC or photobleached FITC. The assay, using the S-r-FITC-1 peptide, gave results comparable to radiotracer experiments.

Key accomplishments

- Synthesis of bombesin peptide derivatives
- Determination of the EC50s of these derivatives vs. [125I-Tyr2] bombesin
- Synthesis of nanoparticle-peptide conjugates
- Development of a non-radiation based assay for uptake and displacement studies

Conclusions

- The GRP receptor binding pocket is surrounded by an area of negative charge.
- An HPLC assay is effective for SPDP linker chemistry, but is cumbersome. Attaching a chromophore is a more efficient methodology.
- The HRP-antibody assay is a practical alternative to radiation methods for displacement and uptake experiments.